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**Spatially-resolved localization and chemical speciation of nickel
and zinc in *Noccaea tymphaea* and *Bornmuellera emarginata***

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27 **ABSTRACT**

28 Hyperaccumulator plants present the ideal model system for studying the physiological regulation
29 of the essential (and potentially toxic) transition elements nickel and zinc. This study used
30 synchrotron X-ray Fluorescence Microscopy (XFM) elemental imaging and spatially resolved X-
31 ray Absorption Spectroscopy (XAS) to elucidate elemental localization and chemical speciation of
32 nickel and zinc in the hyperaccumulators *Noccaea tymphaea* and *Bornmuellera emarginata*. It turns
33 out that in the leaves of *N. tymphaea* nickel and zinc have contrasting localization, and whereas
34 nickel is present in vacuoles of epidermal cells, zinc occurs mainly in the mesophyll cells. In the
35 seeds Ni and Zn are similarly localized and strongly enriched in the cotyledons in *N. tymphaea*. Ni
36 is strongly enriched in the tip of the radicle of *B. emarginata*. *Noccaea tymphaea* has an Fe-rich
37 provascular strand network in the cotyledons of the seed. The chemical speciation of Ni in the intact
38 seeds of *N. tymphaea* is unequivocally associated with carboxylic acids, whereas Zn is present as
39 the phytate species. The spatially resolved spectroscopy did not reveal any spatial variation in
40 chemical speciation of Ni and Zn within the *N. tymphaea* seed. The dissimilar ecophysiological
41 behaviour of Ni and Zn in *N. tymphaea* and *B. emarginata* raises questions about the evolution of
42 hyperaccumulation in these species.

43
44 **Key words:** *capsule, cotyledons; hyperaccumulator; seed; translocation.*

45

1. INTRODUCTION

Hyperaccumulators are unusual plants that accumulate particular metals or metalloids in their living tissues to levels that may be hundreds to thousands of times greater than is normal for most plants^{1,2}. Hyperaccumulator plants have the unique ability to take up and detoxify exceptional concentrations of metals without any signs of toxicity. Plants have been found to hyperaccumulate a wide range of elements, including nickel (Ni), manganese (Mn), cadmium (Cd), copper (Cu), cobalt (Co), selenium (Se), arsenic (As), thallium (Tl) and Zn^{2,3}. This contrasts with 'normal plants' that have a tightly controlled regulation of essential transition elements (Cu, Fe, Ni, Mn, Zn) to avoid either deficiency or toxicity. Hyperaccumulator plants represent the most extreme example of adaptation to a surplus of metal transition elements in their environment, and are therefore ideal model systems for understanding the physiological regulation of essential and potentially toxic, non-essential transition elements^{4,5,6}. In many Ni hyperaccumulator species, Ni occurs in mixtures of citrate and malate complexes that vary in different parts of the plants^{7,8,9}. Hyperaccumulation results from adaptations of the metal regulation mechanisms shared by all higher plants¹⁰. Hence, insights into the mechanisms of hyperaccumulation may be applied to improve the uptake and accumulation of deficient elements, such as iron (Fe) and zinc (Zn), in economically important food crops. These insights may also be applied to limit uptake of potentially phytotoxic elements, such as nickel (Ni) in food crops. The extreme metal accumulation capability of hyperaccumulator plants spawned the concept of phytoextraction for remediating contaminated soils, which has attracted much research effort¹¹. Hyperaccumulator plants also have potential for use in phytotechnologies such as biofortification, phytoremediation and phytomining, the latter utilizes hyperaccumulators as 'metal crops' to sequester Ni or other metals in harvestable biomass that can then be used to produce fine Ni chemicals^{12,13}.

Nickel is the most recent element shown to be essential for higher plants^{14,15}, due to its key role for the activity of urease, an enzyme widely distributed in higher plants¹⁶, and playing a crucial role in nitrogen remobilization from senescing leaves and during seed germination. However, excess Ni induces oxidative and genotoxic stresses that are deleterious to plant growth¹⁷. Therefore, every plant species needs to regulate Ni homeostasis according to its needs. Apart from its function in urease activation, other physiological functions of Ni are poorly understood in higher plants^{6,18}. Although Ni is an essential micronutrient, its physiological requirement is extremely low. It is shown that 0.1 mg Ni kg⁻¹ is sufficient for seed germination and plant growth^{15,19}. Hence, Ni deficiency in naturally-grown plants rarely occurs, and the only known case is for pecan²⁰. The molecular mechanisms involved in the regulation of Ni homeostasis are not well known even in

81 model species such as *Arabidopsis thaliana*. Nickel can be transported from the soil and inside
 82 plants by several families of metal transporters (e.g. ZIP/IRT, IREG, YSL^{21,22,23}). Since plants
 83 normally only require minute amounts of Ni, no Ni specific transporter has so far been identified to
 84 account for enhanced Ni uptake from soil. In non-accumulator species, this function is most likely
 85 performed by one of the ZIP family Zn/Fe/Mn uptake transporters e.g. the Zn-deficiency induced
 86 expression of AtZIP4 can be repressed by supplying Ni²⁺. Preliminary results suggest that one of
 87 these transporters has developed more affinity for Ni²⁺ in Ni hyperaccumulators than in non-
 88 accumulators^{21,23}. The transporter TgMTP1 of the CDF family was originally suspected to mediate
 89 Ni storage in the vacuole of the Ni hyperaccumulator *Noccaea goesingense*²⁴. More recent studies
 90 suggested that transporters of the IREG/Ferroportin family, localized on the vacuolar membrane,
 91 are involved in the storage of Ni in non-accumulators and hyperaccumulators^{21,25}. Studies on Zn
 92 and Cd hyperaccumulation in Brassicaceae species (e.g. *Arabidopsis halleri* and *N. caerulescens*)
 93 revealed that Zn and Cd hyperaccumulation traits are correlated with high and constitutive
 94 expression of genes involved in metal transport, in the biosynthesis of metal chelators and in
 95 cellular defences to oxidative stresses^{26,27,28}. These changes in gene expression are often the
 96 combined effect of gene duplication and altered promoter activity^{29,30}.

97
 98 The genus *Noccaea* has at least 23 species that hyperaccumulate Ni, a further 10 that
 99 hyperaccumulate Zn, three that hyperaccumulate Cd and one that hyperaccumulates Pb^{31,32,33,34,35}.
 100 *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey. (*Thlaspi caerulescens* J.Presl & C.Presl) is
 101 unique in consisting of different ecotypes with distinct metal tolerance and hyperaccumulation
 102 abilities³⁶. While calamine, ultramafic and non-metallicolous populations can hyperaccumulate Zn
 103 and Ni, or Cd, when supplied³⁶, they differ in their ability to tolerate these metals, often depending
 104 on the metal concentrations at their site of origin^{37,38}. Zinc is taken up by ZIP-like plasma
 105 membrane located Zn-transporters. Rather than storing excess Zn in vacuoles of root cells, which
 106 most non-accumulator species do, the Zn²⁺ is loaded into the xylem, by HMA4, as in *Arabidopsis*
 107 *halleri* (L.) O'Kane & Al-Shehbaz²⁹, thus translocated to the leaves, where it is stored in mesophyll
 108 and epidermal vacuoles. Ultramafic populations are known³⁹ and converted into genetically
 109 homogeneous lines by recurrent inbreeding for characterization of Zn, Ni and Cd accumulation and
 110 tolerance properties. The variation in metal tolerance and hyperaccumulation is heritable and
 111 independent of each other^{40,41,42}. The calamine and non-metallicolous populations have been
 112 investigated in more detail than the ultramafic populations. So far most of the analysis of ultramafic
 113 *N. caerulescens* involved the accession from Monte Prinzera, in the Italian Apennine mountain
 114 range³⁶. This accession has been subject to several proteomics studies, trying to correlate local
 115 adaptation to specific protein expression⁴⁴ and was included in transcriptome studies as well⁴⁵. Ni,

and also Zn, xylem loading in *N. caerulescens* is facilitated by high histidine levels⁴³ although the genes involved have not yet been identified.

Noccaea tymphaea (Hausskn.) F.K.Mey. (synonyms: *Thlaspi tymphaeum* Hausskn. and *Thlaspi goesingense* Halácsy) is distributed in Albania, Bosnia and Herzegovina, Greece, Macedonia. It occurs on montane ultramafic soils, where it can accumulate high foliar Ni (up to 11 800 $\mu\text{g g}^{-1}$) and relatively low foliar Zn (up to 179 $\mu\text{g g}^{-1}$)³¹. *Bornmuellera emarginata* (Boiss.) Rešetnik (synonyms: *Leptoplax emarginata* (Boiss.) O. E. Schulz, *Peltaria emarginata* (Boiss.) Hausskn.)⁴⁶ is endemic to ultramafic soils in Greece, with a discontinuous distribution from Pindus mountains, Mt. Smolikas, and the island of Euboea. Some specimens were also sampled in Syria and are kept at Paris Herbarium (P) showing a disjunct distribution pattern across the Eastern Mediterranean. It is a strong Ni hyperaccumulator that can accumulate up to 34 400 $\mu\text{g g}^{-1}$ foliar Ni⁴⁷.

Currently little is known on storage and acquisition of Ni in seeds and during germination. In *Noccaea praecox* (Wulfen) F.K.Mey. (synonym: *Thlaspi praecox* Wulfen.) Cd was mobilised to the shoots during germination, but not to the roots⁴⁸. Scanning electron microscopy with energy dispersive spectroscopy (SEM-EDS) was undertaken on the seeds of *Noccaea pindica* (Hausskn.) Holub (synonym of *Thlaspi pindicum* Hausskn.) and the results showed that Ni accumulated in the micropylar area opposite the radicle and in the epidermis of cotyledons⁴⁹. Little information is available about the elemental distribution in other hyperaccumulating genera of the Brassicaceae, and no study yet has focussed on *B. emarginata* except the SEM-EDS observation of herbarium specimen air-dried leaves which showed accumulation of Ni in the epidermis cells except in the vicinity of stomata⁵⁰. Knowledge about the ecophysiology of Ni and Zn in reproductive organs of hyperaccumulator plants is especially scarce. In order to provide a more general view about the ecophysiology of hyperaccumulation, including the reproduction organs and first phases of life of these plants, this study aimed to elucidate the distribution and chemical speciation of Ni and Zn in the seeds and siliques of *N. tymphaea* and *B. emarginata* originating from their native habitats in Greece. X-ray Fluorescence Microscopy (XFM) has substantial explanatory power for advancing the understanding of the ecophysiology of hyperaccumulation⁹. In order to determine the distribution and spatially-resolved chemical speciation of Ni and Zn in both species, we make use of the singular ability of the Maia detector system^{51,52} to perform ultra-rapid X-ray elemental mapping and spatially resolved X-ray Absorption Spectroscopy (XAS) on live/fresh samples.

2. MATERIALS AND METHODS

2.1 Collection of plant tissues and soils

151 Whole, live plants of *N. tymphaea* were collected in Greece (at the Katara Pass, 39°48'00.5"N
152 21°10'60.0"E, altitude 1690 m. a.s.l.) growing in natural ultramafic soils. Intact seeds capsules were
153 collected from *B. emarginata* in their native habitat Greece (near Trigona, 39°47'29"N, 21°25'32"E,
154 altitude 830 m. a.s.l.). The soils of the collection locality are described in detail elsewhere⁵³. The
155 plants were potted in natural soil from the habitat and brought alive to the P06 beamline (PETRA
156 III Synchrotron, DESY Campus, Hamburg Germany) for the experiments described below.

157

158 **2.2 Chemical bulk analysis of tissue samples**

159 Plant tissue samples for bulk chemical analysis were first dried on silica gel and then dried at 70°C
160 for five days in a drying oven. They were subsequently ground and digested using 4 mL HNO₃
161 (70%) in a microwave oven (Milestone Start D) for a 45-minute programme and diluted to 30 mL
162 with ultrapure water (Millipore 18.2 MΩ·cm at 25°C). Finally, they were analysed with ICP-AES
163 (Thermo iCAP 7400) for Cd, Ni, Co, Cr, Cu, Zn, Mn, Fe, Mg, Ca, Na, K, S, P.

164

165 **2.3 Preparation of tissue samples for X-ray fluorescence microscopy**

166 The seeds and seed capsules could be investigated in their native state without any sample
167 preparation. The intact seeds were mounted between two sheets of Ultralene thin film (4 µm)
168 stretched over a Perspex frame magnetically attached to the x-y motion stage at atmospheric
169 temperature (~20°C). However, in order to reveal the internal distribution of Ni, Zn and other
170 elements inside roots, stems and leaves, cross-sections were prepared. The samples were hand cut
171 with a stainless-steel razor blade ('dry knife'), mounted between two sheets of 4 µm Ultralene thin
172 film in a tight sandwich to limit evaporation, and analysed within 15 minutes after excision. X-ray
173 micro-fluorescence was performed at high speed to keep the scan time to a minimum. Since the
174 penetration depth of the X-rays is greater than the thickness of a cell layer, the information obtained
175 from thick sections is a combined distribution originating from more or less superimposed cell
176 layers. The semi-thick sections (~200 µm) correspond to 3–4 cell layers. As such the obtained data
177 do not reveal subcellular distribution, but nevertheless show the tissue-level distribution (*e.g.*
178 epidermal cells, mesophyll, vascular bundles, *etc.*).

179

180 **2.4 X-ray fluorescence microscopy**

181 The X-ray fluorescence microscopy (XFM) experiments were undertaken at beamline P06 at
182 PETRA III at DESY (Deutsches Elektronen-Synchrotron). The undulator beam was
183 monochromatised using either a Si(111) channel-cut crystal or a double-crystal monochromator,
184 depending on beamline mode for each part of the experiment. A Kirkpatrick-Baez mirror pair was
185 used to focus the incident beam. The X-ray flux of the focussed beam was in the order of 10¹⁰

photons/s⁵⁴. X-ray fluorescence was detected using the Maia detector system in backscatter geometry^{52,55}. The large solid-angle (1.2 steradian) of the Maia detector is particularly suited to biological samples such as these as it allows detection of a good proportion of the fluoresced signal, allowing a reduction of the radiation dose and thus reducing potential damage to a specimen⁵¹.

The 2D μ XRF measurements carried out in the microprobe of P06 at DESY were performed with a beam size of 720×780 nm at an incident energy of 11 keV. The single-slice tomography measurements of the *N. tympheae* seed were carried out with a beamsize of 400×450 nm at a photon energy of 15 keV using the same beamline endstation and general setup. Scanning parameters were a step size of 2 μ m, a dwell time of 1 ms and an angular range of 452 projections covering 360° in 2 subscans. As the seed was naturally dehydrated a cryo-stream was not employed. For the XRF 2D and tomography scans, the pixel size chosen was larger than the focused beam size as a result of necessary compromises due to time constraints.

2.5 Synchrotron X-ray Absorption Spectroscopy (XAS)

Ni and Zn K-edge XAS spectra of the plant tissue samples and standards were recorded in fluorescence mode with the Maia detector. The X-ray beam energy was calibrated using either a Ni or Zn metal foil recorded in transmission, where the first peak of the first derivative was assumed to be 8333 or 9659 eV, respectively. The seeds ($\sim 1.5 \times 0.3$ mm) were scanned at 1.6 μ m pixels with a 20 ms per pixel dwell time. In addition to these μ XRF elemental images on these seeds, spatially resolved XANES spectra were collected as image ‘stacks’ of μ XRF maps, each with 15 μ m pixels and a 12 ms per pixel dwell time, at 170 increasing energies, spanning the energy range 8183–9082 eV over the Ni K-edge at 8.333 keV, and spanning the energy range 9486–9858 eV over the Zn K-edge at 9659 eV.

Several Ni²⁺ and Zn²⁺ standards were prepared by adding organic ligands in calculated molar excess (1:5) to Ni²⁺ and Zn²⁺ to ensure the formation of organo-metallic complexes. The selection of the ligands was based on previous reports of Ni and Zn complexation in hyperaccumulator plants^{7,9,57,58}. Aqueous standards were prepared from Ni(NO₃)₂ or Zn(NO₃)₂ salts respectively in ultrapure water (Millipore) with the following ligands: malate, citrate, oxalate, phytate, and histidine. Supplementary references from previous experiments were also used to increase the number of reference spectra, such as Ni in aqueous solution (Ni-aqueous), Ni-citrate with a metal:ligand ratio equal to 1 (Ni-citrate) and Zn sulfate. The solutions were diluted to 10 mM [Ni or Zn²⁺] before analysis. The pH of the standards was checked, and adjusted to 6. The aqueous standards were then applied to filter paper (Whatmann), allowed to dry and enveloped in Kapton tape before scanning.

221

222

2.6 Data processing and analysis

The XRF event stream was analysed using the Dynamic Analysis method^{59,60} as implemented in GeoPIXE^{61,62}. GeoPIXE provides quantitative first-order self-absorption corrected maps of projected areal elemental density – maps of elemental content. Conversion of X-ray counts to concentration was performed through analysis of Ni and Co XRF reference foil scans (Micromatter, Canada). The samples were each considered of a uniform thickness and either hydrated or dry, with respective thicknesses and compositions of 1000 µm and $C_{7.3}O_{33}H_{59}N_{0.7}S_{0.15}$ for the (hydrated) whole leaf and leaf, stem and root cross-sections, and 1500 µm and $C_{31}O_{15}H_{51}N_2S_{0.8}$ for the (dry) whole seeds and capsules. Assuming a uniform thickness for the seeds introduces further approximations to the measurements of seeds and seed capsules, however, as these have a generally flattened oblate cross-section, the approximation was considered appropriate.

Reconstruction of the single-slice tomographic data was performed using a maximum-likelihood expectation-maximization (MLEM) algorithm.

PCA analysis was performed on the XANES stacks using the MANTiS package. The extracted XANES data were reduced using standard normalization procedures performed with Bruce Ravel and Matthew Newville programs ATHENA and ARTEMIS^{63,64}. Spectra were background subtracted and normalized. The XANES signals obtained were fitted as linear combinations of the standard spectra collected on solutions to evidence the main organic ligands involved in metal complexation. The number of standards was constrained to be at a maximum of three. The sum of components was released and not forced to be equal to 1. The selection of the linear combination was made on the basis of the indicators of fitting quality (χ^2 , r-factor and reduced χ^2), and the number of components was finally set to 2 as a third component did not improve the fitting quality.

3. RESULTS

3.1 Localization of Ni and Zn in *N. tympheae* root, stem and leaf cross-sections

Elemental maps of the root cross-sections (Fig. 1) show that Ni is concentrated mainly in the epidermis, in phloem bundles and pericycle. Zinc is mainly enriched in the phloem bundles. In the stem cross-section (Fig. 2) Ni is enriched in the collenchyma and the phloem bundles, as well as in the xylem.

In the whole leaves of *N. tympheae*, Ni is mainly localised in the leaf blade, with increasing concentrations towards the margins. The whole leaf elemental map of Ni (Fig. 3 and Suppl. Fig 1)

provides intriguing insight in the distribution of Ni at the tissue-level. The circular features are suggestive of major enrichment in the apoplast surrounding large epidermal cells. In contrast, Zn is distributed primarily surrounding the secondary vasculature across the leaf blade. Calcium is clearly depleted in the veins, but high in the interveinal regions. The distribution of Co (not shown) mirrors that of Ni, with enrichment in the leaf margins, and towards the leaf apex. Potassium is enriched throughout the leaf blade, but highest near the petiole.

In the leaf cross-section of *N. tymphaea* Ni and Zn have contrasting localizations (Fig 4 and Suppl. Fig 2). Whereas Ni is present in vacuoles of epidermal cells, Zn occurs mainly in the mesophyll cells, especially on either side of the central vascular bundles (Fig. 4). Calcium is depleted in the vascular bundles, but strongly enriched in the mesophyll and also in the epidermal region. Potassium is enriched in the epidermal cells and in the phloem bundles of the primary vein and also in secondary veinlets.

3.2 Localization of Ni and Zn in seed capsules of *N. tymphaea* and *B. emarginata*

The intact *N. tymphaea* siliques contain 8–10 seeds attached to the central ovary (Fig. 5). Nickel is strongly enriched in vascular bundles of the ovary connected via the hilum onto the seeds. Nickel occurs also at the base of the style (micropyle). Zinc is highest in the micropylar region, in the vascular bundles, and in the seeds in radicles. Nickel and Zn appear to be similarly highly enriched in the cotyledons (Fig. 7). Calcium is highest in the margins of the seed capsules and in the style, whereas K is highest in the central vascular bundles of the ovary. These patterns are similar in another *N. tymphaea* silique (Supp. Fig. 3), but Ni and Zn differ in that Zn is particularly accumulated in the radicles of the seeds, whereas Ni is more broadly enriched in the seeds. The distribution of Co (not shown) again mirrors that of Ni with enrichment mainly in the vascular bundles of the ovary, and in connecting tissues to the seeds.

The intact silique of *B. emarginata* contains just a single seed (Fig. 6 and Suppl. Figs. 4 and 5). Nickel is strongly enriched on the outer margins of the capsules, as well as in the micropylar region and style. Nickel appears enriched in the whole of the seeds, as well as in the hilum. As *B. emarginata* is not a Zn hyperaccumulator, the Zn content is low and its distribution is unremarkable. Calcium is strongly enriched in a peripheral region around the seed margin, likely the wings. Finally, K is especially high in the hilum and vascular bundles leading into the seed capsule and seed.

3.3 Localization of Ni and Zn in the seeds of *N. tymphaea* and *B. emarginata*

The distribution of Ni and Zn is similar and strongly enriched in the cotyledons in *N. tymphaea* as shown in the 2D maps (Fig. 7 and Suppl. Fig. 6). In contrast, in *B. emarginata* they are strongly enriched in the tip of the radicle (Fig. 8). The enrichment of Fe in the provascular strands of the cotyledons and in the hypocotyl) is clearly visible in the network, and Fe is highest in hotspots in the hilum of the micropylar area.

The tomographic reconstructions of the *N. tymphaea* seeds confirm the observations from the 2D maps and show that Ni is localised in the vacuoles (the round solid outlines of vacuoles are clearly visible) of the cotyledon epidermal cells, and similarly in the epidermal cells of the hypocotyl (Fig. 9). The virtual slice is looking tangentially showing the two cotyledons on either side (*i.e.* from the narrow plane) and the hypocotyl on the top right (Suppl Figs. 7, 8 and 9). Nickel is also enriched in the testa (seed coat). Nickel is depleted in the vascular bundles in the cotyledons. In contrast, Zn is enriched more or less evenly throughout the cotyledons, albeit slightly higher in the hypocotyl. It cannot be ascertained whether Zn is present on the vacuoles. The distribution of Fe in the provascular strands (note ‘hollow’ features) of the hypocotyl and cotyledons is clearly visible.

3.4 Spatially-resolved chemical speciation of Ni and Zn in the seeds of *N. tymphaea*

The chemical speciation of Ni in the intact seeds of *N. tymphaea* is unequivocally associated with carboxylic acids (Fig. 10). The spectra extracted from the different regions of the seed (regions determined on the basis of PCA) were strictly identical to each other. Qualitative comparison with reference spectra suggested the predominance of Ni-malate species, confirmed by the linear combination results (Ni evidenced being at 80% complexed with malate). A smaller contribution of Ni-histidine complex can be discerned with the fitting. In the case of Zn (Fig. 11), Zn-phytate species were dominating Zn XANES spectra. In both Ni and Zn, spatially resolved spectroscopy did not reveal any spatial variation in chemical speciation within the seed.

4. DISCUSSION

Until recently detection systems for synchrotron XFM were not sufficiently fast to analyse fresh and hydrated plant tissue because the long dwell times caused excessive radiation damage⁶⁶. The unparalleled ability of the Maia X-ray detection system to undertake very fast measurements (per-pixel dwell times as low as 1 ms and total scan times of less than 20 minutes for leaf cross-sections) makes it possible to analyse live plants and fresh plant materials⁶⁵. There are limitations to this approach, however, including the fact that the elemental maps give information from different

328 depths combined into one plane (in the case of whole plant leaves). The only approach that avoids
329 most or all sample preparation artefacts are cryotechniques which preserve both the distribution, the
330 chemical form and the concentration of all elements *in situ*⁶⁶. However, such techniques are not
331 always available or not operable due to technical constraints, for example a cryo-stream can only
332 cool samples smaller than 2 mm in diameter, and only one synchrotron facility (the BioNano
333 Probe⁶⁷) has a fully enclosed cryogenic chamber for large samples. In order to map elemental
334 distribution within tissues, cross-sectioning is necessary, either physical or virtual by using
335 tomographic methods. Sectioning of fresh plant material using a ‘dry knife’ method (as done in this
336 study) avoids the loss of water-soluble ions (Ni^{2+} , Zn^{2+}), but may result in smearing of cell sap over
337 the sample surface. Such artefacts were, however, not observed in this study and the elemental maps
338 show intact inflated vacuoles (interpreted from the K maps). Seeds and seed capsules are unique
339 among plant organs/tissues in that they are inherently dehydrated, and therefore, can be analysed “as
340 is” in microanalytical experiments.

341

342 Previous studies on the distribution of Ni and Zn in hyperaccumulator plants have shown that in
343 *Hybanthus floribundus* subsp. *adpressus* (Violaceae) seeds the highest Ni concentrations were in
344 the cotyledons, followed by the embryonic axis. In *Pimelea leptospermoides* (Thymelaeaceae)
345 seeds Ni was preferentially localised in the embryonic axis, and in *N. caerulescens*, Zn was highest
346 in cotyledons⁶⁸. Nickel was also concentrated in the epidermis of the cotyledons in *N. caerulescens*
347 seeds⁶⁹, whereas in *N. pindica* seeds Ni was concentrated in the micropylar area and in the
348 epidermis of cotyledons⁵⁰. In *Stackhousia tryonii* (Celastraceae) seeds the highest Ni concentrations
349 were in the pericarp⁷⁰. In *Pycnanandra acuminata* (Sapotaceae) seeds the highest Ni concentration
350 were in the endosperm and mesocarp⁷¹. Similarly, in *Biscutella laevigata* (Brassicaceae) seeds, the
351 highest concentration of Zn was in the endosperm⁷². Finally, in *Berkheya coddii* (Asteraceae) Ni
352 was localised in the lower epidermis, margins of cotyledons, and the pericarp in the micropylar
353 area^{73,74}. The diversity in location of Ni and Zn seeds of various hyperaccumulator plants reflects
354 the variety of phylogenetic origins and distinct physiologies of hyperaccumulator plants. In this
355 study we observed Ni to be localised in the vacuoles of the cotyledon and hypocotyl epidermal cells
356 in *N. tympphaea* seeds, which agrees with the findings for other *Noccaea* species previously studied.
357 During germination the seedling relies primarily on its Fe stores before it develops a root to take up
358 Fe from the soil⁷⁵. *Arabidopsis thaliana* stores Fe in vacuoles of the root endodermis and around the
359 pro-vasculature in the cotyledons⁷⁶. The Fe-rich provascular strand network of the cotyledons
360 occurs in *N. tympphaea* too.

361

362 The European *Noccaea caerulescens* is among the most intensively-studied hyperaccumulator taxa
 363 globally, and used as a model for the genetics, ecology and molecular biology of metal
 364 hyperaccumulation^{38,39,45,77}. The other taxa in the genus, such as *N. goesingense* and *N. praecox*, but
 365 also the various taxa in the *Alyssum* genus, are much less studied^{79,80, 81, 82}. *N. tymphaea*, and taxa
 366 from other genera (*B. emarginata*), have so far obtained little attention, mostly because they grow in
 367 a rather confined and remote area in the Balkan region of Europe. Most *Noccaea* species can
 368 hyperaccumulate Zn, whereas many taxa also hyperaccumulate Ni^{5,83,84}, but since most species have
 369 only been sampled in the field and not been re-grown on Zn or Ni containing soil, this issue is far
 370 from resolved. We set out to determine whether the distribution and chemical speciation of Ni and
 371 Zn differed in *N. tymphaea* and *B. emarginata*, both sampled from ultramafic soil. Although we
 372 expected important differences, due to the differing physiological functions (potentially toxic for Ni
 373 and essential for Zn) of these elements, the results show that Ni and Zn behave remarkably similar
 374 in *N. tymphaea* and *B. emarginata*. The chemical speciation of Ni is univocally associated with low
 375 molecular weight carboxylic ligands (likely malate), as in most hyperaccumulator species studied to
 376 date^{7,9,58,85}. Specifically, in *N. caerulescens* and *B. emarginata* X-ray absorption spectroscopy
 377 showed that citrate was found as the predominant ligand for Ni in stems, whereas in the leaves
 378 malate was predominant⁷. In contrast, Zn was associated with phytates in the seeds. In the
 379 ultramafic soils of which *N. tymphaea* and *B. emarginata* grow, Ni is present at 20–50-fold higher
 380 concentrations, which explains concentrations differences in the plant shoots. Under these
 381 conditions *N. tymphaea* is not a Zn hyperaccumulator (foliar Zn reaches up to 362 $\mu\text{g g}^{-1}$). Based on
 382 the predominant Ni and Zn hyperaccumulation properties found in the current *Noccaea* spp., we
 383 hypothesize that the genus evolved from a Ni adapted and probably Ni hyperaccumulating ancestor.
 384 Some species managed to escape from ultramafic soil and develop as Zn hyperaccumulators on
 385 non-metallicolous soils, with a few, e.g. *N. caerulescens* later adapting to and (re-)colonizing
 386 calamine and ultramafic soils. *Noccaea tymphaea* may represent a taxon that never left the
 387 ultramafic conditions and remained adapted to Ni hyperaccumulation.
 388
 389 This study has shown that XFM can successfully be applied to help answering questions about the
 390 mechanisms of trace element hyperaccumulation, providing elemental distribution and chemical
 391 speciation in fresh/live hyperaccumulator plant tissues. . The dissimilar ecophysiological behaviour
 392 of Ni and Zn in *N. tymphaea* and *B. emarginata* raises questions about the evolution of
 393 hyperaccumulation in these species. Given that Zn accumulation is constitutive in *Noccaea* spp.
 394 occurring in non-metalliferous populations, Ni hyperaccumulation may have evolved as an
 395 adaptation when plants colonised ultramafic soils. In comparison, *B. emarginata* is not a Zn
 396 hyperaccumulator, and only hyperaccumulates (Ni) on metalliferous soils.

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713

714 **FIGURE CAPTIONS**

715

716 **Figure 1.** Elemental μ XRF maps of fresh *Noccaea tymphaea* root hand cut section. The maps
717 measure 4.6×3.2 mm (460×316 pixels). The elemental image was acquired in 10- μ m step size
718 with 5 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps. Abbreviations
719 annotated of anatomical features: *C* cortex, *Xy* xylem, *Ph* phloem.

720

721 **Figure 2.** Elemental μ XRF maps of fresh *Noccaea tymphaea* stem hand cut section. The maps
722 measure 1.72×1.78 mm (430×444 pixels). The elemental image was acquired in 4- μ m step size
723 with 7 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps. Abbreviations
724 annotated of anatomical features: *C* cortex, *Xy* xylem, *Ph* phloem.

725

726 **Figure 3.** Elemental μ XRF maps of fresh *Noccaea tymphaea* whole mature leaf. The maps measure
727 12.55×9.28 mm (502×371 pixels). The elemental image was acquired in 25- μ m step size with 10
728 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

729

730 **Figure 4.** Elemental μ XRF maps of fresh *Noccaea tymphaea* leaf hand cut section. The maps
731 measure 4.45×0.91 mm (890×181 pixels). The elemental image was acquired in 5- μ m step size
732 with 12m ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.
733 Abbreviations annotated of anatomical features: *UE* epidermis, *LE* epidermis, *PM* palisade
734 mesophyll, *SM* spongy mesophyll, *Xy* xylem, *Ph* phloem.

735

736 **Figure 5.** Elemental μ XRF maps of *Noccaea tymphaea* intact silique. The maps measure $4.91 \times$
737 10.02 mm (327×668 pixels). The elemental image was acquired in 15- μ m step size with 10 ms
738 dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

739

740 **Figure 6.** Elemental μ XRF maps of *Bornmuellera emarginata* intact silique. The maps measure 7.5
741 $\times 6.52$ mm (375×326 pixels). The elemental image was acquired in 20- μ m step size with 20 ms
742 dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

743

744 **Figure 7.** Elemental μ XRF maps of *Noccaea tymphaea* intact whole seed. The maps measure 1.83
745 $\times 1.15$ mm (1143×717 pixels). The elemental image was acquired in 1- μ m step size with 20 ms
746 dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

747 **Figure 8.** Elemental μ XRF maps of *Bornmuellera emarginata* intact whole seed. The maps
748 measure 4.52×3.73 mm (903×746 pixels). The elemental image was acquired in 5- μ m step size
749 with 20 ms dwell per pixel, 11.0 keV, incident beam, showing K, Ca, Ni and Zn maps.

750

751 **Figure 9.** Single-slice tomography μ XRF maps of *Noccaea tymphaea* intact whole seed. The
752 elemental image was acquired in 2- μ m step size with 1 ms dwell per pixel, with 15.0 keV as the
753 energy of the incident beam, showing Compton, Fe, Ni and Zn K maps.

754

755 **Figure 10.** Nickel speciation within the *Noccaea tymphaea* seed. A Principal Component Analysis
756 (PCA) was performed on the stack of fluorescence scans, deciphering 4 regions of interest (A and
757 B) from which XANES spectra were extracted. 2 supplementary spectra were extracted from the
758 whole seed (white dotted line on picture A) and from the tip of the hypocotyl (black dotted line on
759 picture A). Panel C shows the corresponding XANES, compared to Ni-malate and Ni-histidine
760 spectra. Panel D displays the linear combination fitting (red dotted line) for one spectrum.

761

762 **Figure 11.** Zinc speciation within the *Noccaea tymphaea* seed. A PCA was performed on the stack
763 of fluorescence scans, deciphering 4 regions of interest (A and B) from which XANES spectra were
764 extracted. 2 supplementary spectra were extracted from the whole seed (white dotted line on picture
765 A) and from the tip of the hypocotyl (black dotted line on picture A). Panel C presents the different
766 spectra and compares them to Zn-phytate spectrum recorded in the same conditions on P06.
767 Spectrum 3 displays a high background level, preventing a correct interpretation and was discarded.
768 Panel D shows the linear combination fitting (red dotted line) and the fitting residual (green line) for
769 one spectrum.

Table 1. Macro and trace element concentrations in roots, stems and flowers of *Bornmuellera emarginata* (values in $\mu\text{g g}^{-1}$ dry weight) with ICP-AES. <LQ is lower than the limit for quantification.

Species	Part	Al	Ca	K	Mg	Mn	Na	P	S
<i>Bornmuellera emarginata</i>	Roots	<LQ	7900	11 325	1818	32	5570	1068	3559
	Roots	875	6349	10 070	3335	73	5416	1871	6492
	Stems	<LQ	14 430	23 535	3576	51	3037	2806	10 735
	Stems	1336	4583	13 350	4565	212	3669	1632	4531
	Flower	<LQ	21 010	15 350	6154	95	<LQ	2372	13870
Species	Part	Fe	Mn	Zn	Co	Cr	Cu	Ni	Zn
<i>Bornmuellera emarginata</i>	Roots	62	32	193	2.1	1.1	18	539	200
	Roots	1743	73	177	8	43	35	289	166
	Stems	43	51	251	4.9	1.0	20	1030	259
	Stems	2936	212	<LQ	16	66	21	499	90
	Flower	<LQ	95	311	43	<LQ	47	3641	306

Table 2. Macro and trace element concentrations in leaves, fruits, and seeds of the actual *Noccaea tymphaea* and *Bornmuellera emarginata* samples used for XFM elemental mapping (values in $\mu\text{g g}^{-1}$ dry weight) with ICP-AES. <LQ is lower than the limit for quantification.

Species	Organ	P	S	Mg	K	Ca	Fe	Mn	Zn	Ni
<i>Noccaea tymphaea</i>	Leaves	616	2284	3496	4059	9549	218	26	362	12 410
<i>Bornmuellera emarginata</i>	Fruits	1639	8564	2996	7742	6317	38	7.8	96	10 345
<i>Noccaea tymphaea</i>	Seeds	3834	5738	1628	6087	3701	<LQ	50	112	2504
<i>Bornmuellera emarginata</i>	Seeds	2926	15 159	1775	7800	4419	24	12	69	6242